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DOI:

[10.1111/odi.12410](https://doi.org/10.1111/odi.12410)

*Document Version*

Peer reviewed version

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*Citation for published version (APA):*

Moyes, D. L., Islam, A., Kohli, A., & Naglik, J. R. (2016). Oral epithelial cells and their interactions with HIV-1. *ORAL DISEASES*, 22(S1). DOI: 10.1111/odi.12410

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1   **Oral epithelial cells and their interactions with HIV-1**

2   **HIV-1 and the oral epithelium**

3

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9   Key Words: HIV, AIDS, epithelial cells, oral epithelium, infectious disease, mucosal immunity

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## Abstract

As the AIDS pandemic has continued, our understanding of the events that occur during the entry and infection of conventional, susceptible cells has increased dramatically, leading to the development of control therapies for HIV infected individuals. However, an ongoing hole in our understanding is how HIV crosses the mucosal barriers to gain access to permissive cells, despite how important this information would be in developing successful vaccines and other preventative measures such as topical anti-HIV microbicides. In particular, our knowledge of the role that epithelial cells of the mucosal surfaces play in infection – both during early phases and throughout the life of an infected individual, is currently hazy at best. However, several studies in recent years suggest that HIV can bind to and traverse these mucosal epithelial cells, providing a reservoir of infection that can subsequently infect underlying permissive cells. Despite this interaction with epithelial cells, evidence suggests HIV-1 does not productively infect these cells, although they are capable of transferring surface-bound and transcytosed virus to other, permissive cells. Further, there appear to be key differences between adult and infant epithelial cells in the degree to which HIV can transcytose and infect the epithelium. Thus, it is clear that, whilst not primary targets for infection and virus replication, epithelial cells play an important role in the infection cycle and improving our understanding of their interactions with HIV could potentially provide key insights necessary to develop effective preventative therapies.

## 29    **Introduction**

30    Despite a reduction in the number of articles about the HIV epidemic in the Western press over the  
31    last two decades, HIV infection is a major ongoing issue in global health. Recent epidemiology  
32    figures indicate that at the end of 2014 there were approximately 37 million people living with HIV.  
33    During 2014, approximately 2 million fresh cases of infection were reported, whilst around 1.2  
34    million people died of HIV/AIDS related illness (UNAIDS, 2015). This, however, could be just the tip of  
35    the iceberg. Estimates suggest that only about 50% of cases are reported, implying that the true  
36    figures for infection are much higher. Although the introduction of HAART therapy has been a bright  
37    spot in the landscape of the HIV Pandemic (along with other measures, the era of HAART therapy  
38    has seen a 42% drop in annual mortality in 2014 since its peak in 2004), we are currently still no  
39    closer to developing effective therapies such as vaccines and topical anti-HIV microbicides that could  
40    be used to prevent infection in the first place. A key step in the fight to develop these therapeutics is  
41    improving our currently poor understanding of HIV infection at mucosal surfaces.

42    The primary infection route for HIV-1 is across mucosal surfaces such as the gastrointestinal and  
43    genital tracts (Hladik & McElrath, 2008), with exposure occurring during events such as sexual  
44    activity and breast-feeding. Currently, the majority of new infections occur as a result of  
45    heterosexual transmission in adults, with HIV-1 having been isolated from both seminal fluid and  
46    cervicovaginal secretions (Coombs et al., 1998, Dulioust et al., 1998, Goulston et al., 1998). In  
47    infants, however, infection by HIV-1 typically occurs as a result of breast-feeding, with the oral and  
48    oropharyngeal tissues representing the initial exposure sites. Effective transmission across genital  
49    mucosal surfaces (in particular, the vagina, ecto-and endocervix and uterus) is assisted by the  
50    presence of HIV-1 target cells within these surfaces, such as dendritic cells, macrophages and  
51    lymphocytes (Yeaman et al., 1998, Ballweber et al., 2011, Shen et al., 2011, Kaldensjo et al., 2011,  
52    Hladik et al., 2007, Miller & Shattock, 2003, Bhoopat et al., 2001, Hu et al., 2004, Maher et al., 2005).  
53    Indeed, it has been established that dendritic cells can sample mucosal surfaces such as the gut

directly, without needing antigens to be transported through the epithelial layer (Niess et al., 2005). However, a subset of dendritic cells, the Langerhans cells, has been shown to have an anti-HIV function, mediated by the Langerin surface receptor (de Witte et al., 2007), and these cells are known to be present in oral mucosa (Hussain & Lehner, 1995). The key role of both dendritic and Langerhans cells in HIV infection is extensively reviewed elsewhere (van den Berg & Geijtenbeek, 2013). Despite the apparent ease with which HIV crosses the genital and rectal mucosa, transmission across the oral mucosa is thought to be rare (Milman & Sharma, 1994, Janoff & Smith, 2001, Wilkinson & Cunningham, 2006, Cohen et al., 2000, Rothenberg et al., 1998, Jotwani et al., 2004), with several mechanisms (such as neutralizing antibodies, innate anti-HIV salivary factors, presence of Langerhans cells and the epithelium) proposed to explain this phenomenon (Challacombe & Naglik, 2006, Herzberg et al., 2006, Kazmi et al., 2006, Malamud & Wahl, 2010, Weinberg et al., 2011). Despite this, oral transmission can occur. For example, SIV transmission across non-traumatised oral epithelia, resulting in dissemination and systemic infection in primates has been reported in several studies (Baba et al., 1996, Herz et al., 2002, Joag et al., 1997, Milush et al., 2004). As a result, the epithelium may represent either a physical barrier against direct infection or a potential reservoir to aid infection. The importance of determining which of these is prevalent is of particular importance, given the potential for viral transference in nursing infants or during oro-genital contact in adults.

## **Initial contact with oral mucosa**

The initial contact between the host and HIV-1 in the oral cavity occurs between virus and the oral and oropharyngeal mucosal tissues (Herzberg et al., 2006). However, given the variety of cell types, along with the preponderance of epithelial cells in these tissues, neither the primary infected cell type, nor the initial site of infection has as yet been identified. It has become evident, however, that upon exposure of these sites to the virus, an anti-HIV response is generated. For example, after unprotected oro-genital contact with an HIV<sup>+</sup> partner, uninfected men have been shown to mount a

salivary IgA1 response that neutralises the HIV-1 virions (Hasselrot et al., 2009), thus demonstrating that far from remaining ignorant of the presence of the virion, these surfaces are capable of recognising and responding to HIV-1. What is unclear is where the response is initiated. Are these responses a result of viral interaction with and infection of resident infectable immune cells such as langerhans dendritic cells or macrophages or, more controversially, are they instigated through recognition of viral particles by the epithelial cells that make up the majority of these surfaces? On the face of it, this seems like an easy answer - epithelial cells are not known for their expression of the canonical receptors for HIV - namely CD4, CCR5 and CXCR4, so they should not be infected or interact directly with the virus. Further, extensive research over the years has identified CD4<sup>+</sup> T cells and macrophages, along with dendritic cells, as being the key target cells of the HIV virus. However, over the last decade, there has been a small but persistent number of studies from different groups suggesting that HIV can interact, associate with or even infect epithelial cells (Vacharaksa et al., 2008, Kohli et al., 2014, Rodriguez-Inigo et al., 2005, Liu et al., 2003, Moore et al., 2002, Moore et al., 2003). These studies suggests that oral epithelial cells themselves may play a key role in the initial contact events in the oral cavity, possibly being the initial point of contact. However, whether the epithelial cells that make up the oral mucosa can serve as a reservoir for HIV-1 virions during initial or subsequent infection events, or even if they can function as a seat of infection remains uncertain (Richman et al., 2009). The importance of the oral mucosa in HIV infection, however, cannot be ignored. Whilst anti-HIV immune responses are generated, augmentation of these responses with topical anti-HIV agents (Li et al., 2009) strongly suggests that infection and evasion of immune responses at the oral mucosae represents a potential route of infection.

#### **Epithelial cells - infected or bound?**

Inherent to the question of whether oral (or other) epithelial cells can be infected by HIV-1, is whether they possess the correct, canonical HIV receptors – CD4 and CXCR4 or CCR5. The target cells of an HIV-1 infection (T cells, dendritic cells and macrophages) all express CD4 and either or both of

the chemokine co-receptors, CXCR4 or CCR5. The events during infection of these cells have been the source of much research and has now been fairly well characterised (Pierson & Doms, 2003). Initial contact between the viral envelope protein gp120 and host cell CD4 leads to a conformational change in gp120 followed by further interaction with one or other of the fusion co-receptors, CCR5 or CXCR4. This leads to gp41-mediated fusion of the viral membrane with the host cell membrane, allowing entry into the cell. Once inside the cell, the HIV-1 RNA genome is reverse transcribed into DNA and integrated into the host cell genome. Thus, it would appear that a central requirement in the infection of a cell is the expression of CD4 and either or both of CCR5 and CXCR4. Studies by several groups have clearly demonstrated that, unlike classical HIV-infectable cells (T cells etc), epithelial cells do not express CD4 (Liu et al., 2003, Kumar et al., 2006, Quinones-Mateu et al., 2003, Vacharaksa et al., 2008, Kohli et al., 2014, Walsh et al., 1990), whilst levels of CXCR4 and CCR5 are either very low or undetectable (Kohli et al., 2014, Liu et al., 2013, Vacharaksa et al., 2008, Cutler & Jotwani, 2006, Giacaman et al., 2008, Berlier et al., 2005, Bobardt et al., 2007, Dezzutti et al., 2001).

Given this apparent lack of HIV-1 binding cellular receptors, the question is how do HIV-1 virions bind to epithelial cells? The answer to this question is via the use of alternative HIV-associative receptors. Over the course of the history of HIV-1 research, several different groups have demonstrated that in addition to the 'canonical' receptors CD4 and CCR5/CXCR4, HIV-1 can also bind to a selection of other cell surface receptors or moieties, utilising substitute mechanisms of binding and cellular entry (**Table 1**). For example, the viral envelope protein gp160 (gp120 and gp41) binds to, among others, DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) (Geijtenbeek et al., 2000, van Kooyk & Geijtenbeek, 2003), GalCer (the glycosphingolipid galactosylceramide) (Alfsen & Bomsel, 2002, Delezay et al., 1997, Harouse et al., 1991) and heparan sulphate glycans (HSPGs) including the syndecan family receptors (Bobardt et al., 2003, Wu et al., 2003). Each of these receptors has been shown to play a role in either simply binding the virus and 'passing it on' to target cells (*trans*-infection) or, in some cases, enhance direct infection of the binding cell (*cis*-infection) by an active process as well (e.g. D-SIGN). Most notable of these is the C-

type lectin DC-SIGN. Work investigating the interactions of this lectin with HIV-1 gp120 have indicated that as well as enhancing infection by increasing the concentration of virus on the cellular surface, it also increases the affinity of gp120 for CD4 (Hijazi et al., 2011). Both GalCer (Magerus-Chatinet et al., 2007) and HPSGs (Jiang et al., 2015) have also been shown to play a role in *trans*-infection.

These 'non-canonical' receptor moieties are expressed on a wider range of cell types than CD4 and the chemokine co-receptors. Both GalCer and HSPGs in particular have been shown to be abundantly expressed in oral epithelial cells, although DC-SIGN is not (Kohli et al., 2014). This expression on the epithelial cell component has been proposed as a mechanism promoting the binding and transfer of HIV-1 virions across both the oral and vaginal epithelial layers thus improving/aiding initial infection events (Bobardt et al., 2007, Dezzutti et al., 2001, Weinberg et al., 2011, Wu et al., 2003, Yeaman et al., 1998). Given that these interaction processes between non-canonical receptors and HIV-1 are regarded as being co-receptor independent (CXCR4/CCR5), it is, therefore, somewhat of a surprise that the R5-tropic HIV-1 viruses (those that utilise CCR5 as a co-receptor) appear to be preferentially transmitted across mucosal surfaces (Margolis & Shattock, 2006).

Although there is currently no evidence to indicate that any of these non-canonical receptors can directly initiate *cis*-infection of an epithelial cell in a CD4+/CCR5/CXCR4 independent fashion, there have been studies published that suggest that HIV-1 is capable of infecting and integrating into oral (Vacharaksa et al., 2008) and genitourinary tract (Hladik et al., 2007) epithelial cells, although in both cases, this infection was non-productive - *i.e.* does not result in the production of viral transcripts or progeny virions. However, in a more recent study, we demonstrated using a variety of techniques that whilst both X4- and R5-tropic HIV-1 could bind to oral and vaginal epithelial cells, they were unable to infect - productively or unproductively (Kohli et al., 2014). When analysed, virus treated cells showed not only showed no infective virion production when the virions were pre-treated with



DNase to remove any nucleic acid contamination. They also demonstrated no proviral integration, spliced viral mRNA or *de novo* synthesis of viral proteins. The absence of all these indicators of viral infection and production indicate that although oral and vaginal epithelial cells have a variety of non-canonical HIV receptors on their surface, they appear to be non-permissive to viral infection by the HIV-1 virus. It is notable that this non-permissivity to infection by HIV-1 is not down to an inherent inability of HIV-1 to replicate in epithelial cells. We found that if a VSV-G pseudotyped *gfp* containing HIV-1 virus (guaranteeing entry via the endocytic pathway) was used to infect epithelial cells, then normal proviral integration could occur, with infected cells expressing GFP at similar levels to a permissive cell line. Together with the existence of reporter 'permissive' cell lines that express CD4, CCR5 and/or CXCR4, such as the TZM-bl line, that are based on epithelial cells, this data suggests that if the HIV-1 virion can gain entry to oral epithelial cells in a receptor-independent or other non-conventional mechanisms (such as endocytosis), then this could potentially lead to the production of infectious virions. Thus, it is possible that when conditions arise *in vivo* that facilitate bypassing receptor-mediated entry (e.g. during inflammation events such as gingivitis when cells are damaged, or by 'piggy backing' on another microbe), epithelial cells in the epithelium could support productive HIV-1 infections, potentially by harbouring infectious HIV-1 that is then *trans*-infected to incoming permissive immune cells (Giavedoni et al., 2013).

Despite the inability of HIV-1 to successfully infect epithelial cells, however, we like several other studies found that HIV virions can be bound by epithelial cells, as indicated by the presence of both gp120 and p24 on infected epithelial cells (determined by flow cytometry and immunoblotting), and the presence of HIV-1 RNA genome (determined by PCR on RNA isolated from infected cells). Interestingly, there are difference in the amount of virus bound by epithelial cells from different locations, with the oropharyngeal FaDu epithelial cell line binding more than either a buccal or vulvovaginal cell line. The binding (likely through non-canonical receptors) also proved to be trypsin-sensitive, particularly for the R5 tropic virus (Kohli et al., 2014), suggesting a degree of protein involvement. Thus, from our study and others, it appears that HIV-1 does indeed bind to epithelial

cells, although there are differences between cells from different locations. These differences likely reflect differences in the expression of the different non-canonical receptors on their surfaces, and may well change with the age and infection status of an individual, as well as with any drug regimen the individual is on.

#### **Epithelial cell transfer of HIV-1**

Given that HIV-1 can demonstrably bind to epithelial cells, albeit without any accompanying *cis*-infection, the question arises whether epithelial cells can act as a reservoir in some way for the virus. The hypothesis would be that surface-bound HIV can be transferred from epithelial cells to other, susceptible cells, such as dendritic cells, macrophages and T cells integrated in or underlying the epithelial layer. Although there is a study which was unable to demonstrate successful transfer of infectious HIV-1 to permissive PBMCs (peripheral blood mononuclear cells) (Berlier et al., 2005), there are several studies that have produced evidence to support the hypothesis (Yeaman et al., 1998, Ballweber et al., 2011, Shen et al., 2011, Hladik et al., 2007, Khanna et al., 2002, Miller & Shattock, 2003, Dezzutti et al., 2001, Spira et al., 1996, Hu et al., 2000). With this in mind, we tested whether epithelial cell-bound HIV could be successfully transferred to a reporter cell line using an overlay system, and demonstrated that both R5 and X4-tropic virus were capable of retaining infectivity and being transferred to susceptible cells after being bound by epithelial cells (Kohli et al., 2014). Others have also shown HIV binding activity for oral cells (Liu et al., 2003, Vacharaksa et al., 2008, Wu et al., 2003, Mondor et al., 1998, Roderiquez et al., 1995), which supports the hypothesis that oral epithelial cells can bind HIV-1 and transfer it to other cells, acting as a 'sump' or reservoir for the virus - whether it has been produced locally or has come from an external source. What is not clear at this time is the receptors responsible for this binding. It is clear from studying the effects of trypsin treatment on the levels of binding that there is some protein interaction involved. Likewise, studies examining the effects of heparin or heparin sulfate (blocking binding through HSPGs) indicate that these treatments can also reduce viral attachment (Bobardt et al., 2003, Wu et

al., 2003, Mondor et al., 1998, Roderiquez et al., 1995). However, there is no current definitive proof for one specific moiety on either side being key. The likelihood is that there are a range of receptor/ligand interactions on both virion and host epithelial cell that all play differing roles, depending on the site, environment and other factors.

Binding and transferring the virus is only one part of the story, however. Most of the HIV-1 susceptible cells in mucosal surfaces are present at lower levels than the top of the epithelium. Thus, to be a truly effective source of viral transmission, it is necessary for the virus to be transported through the epithelial layer whilst retaining infectivity. Using transwell cultures, we demonstrated the transcytosis of infectious virus through an oral or vaginal epithelial cell layer, with successful infection of susceptible cells on the basal side of the monolayer (Kohli et al., 2014). Other studies have also shown transcytosis through vaginal epithelial layers (Kinlock et al., 2014, Ferreira et al., 2015). Interestingly, these studies indicate that this is an endocytic pathway dependent mechanism (Kinlock et al., 2014), and that the female hormone medroxyprogesterone acetate boosts the transit and subsequent infection (Ferreira et al., 2015). A further study has also implicated anti-HIV-1 antibody-neonatal Fc receptor interactions as also playing an enhancing role in the transcytosis of HIV across an epithelial cell surface (Gupta et al., 2013). This strongly suggests that host factors are critical in regulating the transport of HIV virions across epithelial surfaces deeper into the layer and thus subsequent infection of susceptible cells in these tissues. More, HIV-1 is able to hijack host mechanisms to boost this event. Investigations using *in vivo* models suggest that HIV can indeed successfully pass through the epithelial layer to permissive cells, although much of this work has been carried out looking at vaginal/genitourinary mucosa (Ferreira et al., 2015; Spira, 1996 #1936, Hu et al., 2000, Baba et al., 1996, Herz et al., 2002, Joag et al., 1997, Milush et al., 2004).

The data obtained from all these studies lead us to develop the hypothesis that the epithelial cells at oral and other mucosal surfaces not only bind cell free HIV, but that they also transport infectious virions deeper into the mucosal layers where they can infect susceptible cells.

#### **Further considerations**

It is clearly apparent that different conditions/environments at mucosal surfaces can materially affect the movement and maintenance of HIV-1 virions within the mucosae. What we have seen here is that there are differences in the transmission rates of epithelial surfaces depending on the status of the epithelial cells or production of host factors. This then leads us to wonder whether the transmission of virus through the oral epithelium varies with the age of the host. This is of particular interest, given the rate of transmission from mother to child. This mother to child transmission (MTCT) can occur during pregnancy, labour and delivery or through breast feeding after birth (Politch et al., 2014). MTCT during pregnancy and labour/delivery occurs in 15-25% of cases unless specific interventions are made, but a further 5-20% of infants will be infected as a result of post-natal breast feeding (Politch et al., 2014, Roskoski, 2014). Given the low incidence of transmission traditionally associated with oral sex (Smith et al., 2005), this suggests that there may be something different about neonatal/foetal oral epithelium that allows a significant number of infectious events due to transmission across the oral mucosa. Two recent studies from Tugizov et al suggest that this might be the case (Tugizov et al., 2011, Tugizov et al., 2012). In these studies, the authors determined that there was a significant number in the amount of HIV virions that successfully traversed the oral epithelium and infected the underlying susceptible cells in fetal/neonatal oral epithelium. In contrast, transmission through adult epithelium was reduced. Further, HIV-1 virions transcytosed through adult oral epithelium showed reduced infectivity, whereas those transcytosed through foetal/neonatal epithelium (Tugizov et al., 2011). The likelihood is that this reduction is due to an increase in the secretion of anti-HIV factors such as  $\beta$ -defensins 2 and 3, as well as secretory

leukocyte protease inhibitor (Tugizov et al., 2011). Thus the age of the host is also a key factor in the transmission of HIV virions across the epithelial surfaces.

## Summary

As we have seen throughout this review, the oral epithelium and the epithelial cells that comprise the majority of these surfaces are not simply a passive barrier during HIV-1 infections. In contrast, they have a significant role to play in infection events as well as potentially acting as reservoirs for infection. We propose that under normal conditions HIV-1 will bind to adult epithelial cells in the oral epithelium, but that this binding will not result in productive infection. However, post-capture, some of these virus particles may be retained and translocated through the epithelial layer to underlying target cells which can then be productively infected, although a large percentage of these virions may be killed or inactivated by host factors. In neonates, however, these host factors play a far more minor role, meaning that transmission across oral epithelium via epithelial cells and subsequent infection of susceptible cells is a far more common event. Thus, further targeting epithelial cells and the epithelium in general, particularly in neonates and infants represents a highly viable preventative therapy to halt the spread of HIV infection.

## Acknowledgments

This work was supported by NIDCR DE017514 (to J.N.) Medical Research Council (MR-J008303-1) (to J.R.N. and D.L.M.) and Biotechnology and Biological Sciences Research Council (BB-J016411-1). (to J.R.N. and D.L.M.)

**Table 1: List of non-canonical HIV receptors.**

| Receptor           | Role  | Reference                 |
|--------------------|---|---------------------------|
| DC-SIGN            | Binds HIV gp120; increases affinity of gp120 for CD4; increases infectivity | (Hijazi et al., 2011)     |
| Galctosyl Ceramide | Binds HIV gp120; allows HIV to bind cells in a CD4 independent fashion      | (Magerus-Chatinet et al., |

|                                |  |                      |
|--------------------------------|--|----------------------|
|                                |  | 2007)                |
| <b>Heparan sulfate glycans</b> | Binds HIV gp120; allows HIV to bind cells in a CD4 independent fashion | (Jiang et al., 2015) |

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